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Asymptomatic hyperchromasia of red blood cells is associated with decreased red cell deformability

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Abstract

Background

Hereditary membranopathies are important red blood cell (RBC) disorders, however, diagnosis can be challenging in mild cases for which osmotic fragility tests yield normal results. The diagnostic gold standard under these conditions is osmotic gradient ektacytometry. Since the diagnosis of hereditary membranopathies influences patient management, a screening test might be of value.

Design and Methods

Since the percentage of hyperchromic RBCs is routinely measured by many hematologic laboratories, we evaluated the predictive value of an elevated percentage of hyperchromic RBCs for the detection of RBC disorders. We did an extensive workup of all patients undergoing standard hematogram during a period of six months at our institution with a fraction of hyperchromic RBC larger than 10% by reviewing the medical history and performing osmotic gradient ektacytometry.

Results

32'226 individuals were screened of which 162 (0.5%) showed more than 10% hyperchromic RBC. All of these patients which could be examined by ektatocytometry showed abnormal membrane deformability. Hereditary spherocytosis (HS) was found in a third of these patients, in most cases unknown to the patient and currently asymptomatic. Another 17.9% of the patients with an elevated subpopulation of hyperchromic RBC suffered from a known viral infection (HIV, Hepatitis).

Conclusions

Our study shows that a proportion of hyperchromic erythrocytes larger than 10% is associated with RBC membrane disorders in a third of patients and further follow up should be considered.

Introduction

Hereditary spherocytosis (HS) is a common form of hereditary red blood cell (RBC) membranopathies. Symptoms range from asymptomatic forms to occasional severe diseases requiring splenectomy and regular transfusions; a large portion of asymptomatic patients are unaware of their condition[1]. Due to the much shortened life time of erythrocytes, HS is a contraindication for blood donation in Switzerland[2] and most developed countries. Therefore, screening for asymptomatic forms of HS might improve the effect of blood transfusions.

HS is typically diagnosed by osmotic fragility testing[3], however, mild forms of hereditary RBC membranopathies with only limited hemolytic activity are often difficult to identify[4]. In these cases, osmotic fragility tests have a poor sensitivity and about 20% of mild cases of HS are missed[5]. In contrast to this, osmotic gradient ektacytometry with the simultaneous recording of deformability against the solute concentration remains the gold standard for the diagnosis of altered erythrocyte deformability[1,6]. However, this assay is technically challenging and therefore not a suitable screening test.

Hyperchromasia of red blood cells has been described in many red blood cell membranopathies, especially HS[7]. While in clinical practice frequently beside the morphology of RBC only the mean cellular hemoglobin concentration (MCHC) is considered, in our experience an elevated percentage of hyperchromic RBC even in the absence of an elevated MCHC can be predictive for an altered RBC membrane[8]. In addition to RBC membranopathies, erythrocytic hyperchromasia can also be caused by autoimmune hemolytic anemia (AIHA), acute oxidant injury, micro- and macroangiopathic hemolytic anemias, hemolytic transfusion reactions, thermal injuries, liver disease, clostridial sepsis, zinc toxicity, poisoning by certain snake, spider and hymenoptera venoms, severe hypophosphatemia, hypersplenism[7], artificial heart valves, massive myocardial infarction and cytotoxic treatment[9]. Furthermore, pseudohyperchromasia due to an artifact of the automatic determination of the RBC indices can be caused by severe hypoosmolality of the plasma[10].

Hyperchromasia might therefore be a sign of a red blood cell membranopathy or a systemic disease. The percentage of hyperchromic red blood has been used before as a screening tool for RBC-membranopathies[11], however, in this study no rigorous follow up of patients was attempted and the diagnostic significance of an elevated percentage of hyperchromic RBCs remains unclear. We therefore started a systematic investigation of all patients with marked elevation of hyperchromic RBC and ektatocytometry was used as a diagnostic tool for RBC-membranopathies. In our study in almost a third of all patients with >10% hyperchromic erythrocytes membranopathies were identified, making the percentage of hyperchromic erythrocytes a useful screening tool for RBC membranopathies and follow up by ektatocytometry worthwhile.

Design and Methods

Comparing the percentage of hyperchromic RBCs and MCHC during two months

In order to evaluate the distribution of the percentage of hyperchromic RBCs and MCHC in our patient population we assessed all patients undergoing standard hematogram at the central hematologic laboratory of the university hospital in Zürich within a period of two months. If samples of a patient were measured on several occasions, only the sample with the highest percentage of hyperchromic RBC was considered. All hematograms where analyzed by an ADVIA 120 (Siemens, Germany). Erythrocytes with a corpuscular hemoglobin concentration of more than 41 g/dl are considered as hyperchromic, whereas the upper limit for the mean corpuscular hemoglobin concentration (MCHC) is defined as 36 g/dl.

Patient recruitment and data collection

From all patients undergoing a standard hematogram during a period of 6 months (February until August 2008), we selected all individuals with 10% or more hyperchromic erythrocytes. The medical history of these patients was reviewed and patients were classified as discussed below (Fig. 2). An elevated proportion of hyperchromic RBC in patients with one ore more known reasons for an acquired disease (see introduction) was considered "acquired". If a hereditary membranopathy such as HS explaining the elevated fraction of hyperchromic erythrocytes had been diagnosed before, the patient was categorized as "known congenital". All other patients were classified as having an "unknown" condition. Medical chart review revealed evidence for viral infections such as hepatitis B or C or human immunodeficiency virus (HIV) in many cases; therefore we divided "unknown" into two subgroups: Patients with documented viral infection and without. If the hyperchromic fraction had been in normal range at least once in the past ("not persistent"), the patient was excluded from further follow up. Even though an occasional presence of a normal fraction of hyperchromic erythrocytes in the patient's history does not exclude a congenital cause with certainty, it at least makes a transient or acquired cause much more likely. For the remaining individuals ("possibly congenital") a work up was done by determining the size of the spleen, performing a Coombs test and osmotic gradient ektacytometry on their RBC. For comparison and as internal controls we also tested several patients with "known congenital" hyperchromasia, documented viral infection and acquired reasons for hyperchromasia.

Osmotic gradient ektacytometry

RBC deformability was studied with an ektacytometer (*Technikon, Bayer, Germany*) in the osmoscan mode as described [6,12-14]. Thereby, deformability of erythrocytes is measured by laser diffractometry. Deformability is plotted against the extracellular osmolality of a viscous solution of 20% dextran that provides a constant shear stress at increasing osmolality. Dextran had an average molecular weight of 70 kDa (*Carl Roth GmbH, Germany*), this solution further

contained 10 mM $NaKHPO_4$ to minimize the effect of pH on deformability[15,] glucose (5.6 mM) and sodium azide (0.4 g/L). The osmolality gradient was obtained by adding sodium chloride to the solution in one compartment of the gradient mixer. The Technikon Ektacytometer measures conductivity of the RBC suspension close to the diffractometer. The system was calibrated beforehand by measuring ektacytometer's conductivity on a series of solutions differing in osmolality by cryoscopic osmometry (*Gonotec Osmomat 030)*. An aliquot of 500 µl whole blood, collected in a standard 10 ml *BD K2H Vacutainer*® (18.0 mg of K_2EDTA), was mixed with 3 ml of isoosmolal dextran solution and directly inserted into the Ektacytometer for measurement of RBC deformability. Blood samples were processed within 1-4 hours after donation and incubated at room temperature prior to mixing with dextran. All samples were measured twice, both values consistently showed an almost perfect correlation. The analog output of the Technikon Ektacytometer was digitalized using a 12 bit A/D-Converter (NI USB-6008, *National Instruments, USA*). On the digitalized osmoscan obtained, the following points were calculated: P_{max} - the point of maximal deformability index (DI) and P_{min} - the point of minimal DI (in the hypoosmolal branch). In addition, the osmolalities where half the DI of P_{max} was reached on both the hypoosmolal branch (P'A) and the hyperosmolal branch (P'B) were recorded (Fig. 3A). In order to compare results from patients and controls, we also recorded and analyzed osmoscans from 30 healthy volunteers. From these data an averaged osmoscan profile was calculated. The respective range (double standard deviation) of the four points P_{min}, P_{max}, P'A and P'B are indicated as boxes in the graphs (Fig. 3). These reference values were used for qualitative and quantitative evaluation of patients' samples.

Determination of P_{min}, P_{max}, P'A and P'B for HS

To acquire discriminatory values from osmoscans of HS patients, we reviewed 15 osmoscans from patients with known HS. Ten of these patients had a spectrin/ankyrin deficiency and five a band 3 deficiency as verified by SDS PAGE (not shown). The osmoscans had been obtained between 1999 and 2004 and were not digitalized but plotted onto paper. Therefore, the four characteristic points were determined manually from the plots.

Ethical approval for this study was granted by the ethical committee of University Hospital Zurich. All authors had access to the primary clinical data.

Results

Comparing the percentage of hyperchromic RBCs and MCHC during two months

In order to define an appropriate screening algorithm for RBC disorders we evaluated standard hematograms analyzed during a period of 2 months at our institution. 38'579 samples of 14'988 individuals were tested. Overall, values obtained for MCHC and the percentage of hyperchromic erythrocytes correlated strongly ($r=0.6$, $p<0.0001$, spearman's rank correlation), Figure 1. Interestingly, this correlation was weaker at the extremes of both parameters (MCHC >36 g/dl and percentage of hyperchromatic RBCs >10%, not shown), confirming that MCHC and percentage of hyperchromatic RBCs cannot be used interchangeably. Of note, only 63% of the 40 patients with more than 10% or more hyperchromatic RBCs had an MCHC above the cut-off. Vice versa, only 14% of the 250 patients with a MCHC >36 g/dl had a percentage of hyperchromatic RBCs of 10% or above. Importantly, the frequency distribution of MCHC and the percentage of hyperchromatic erythrocytes was very different at the positive extremes of both curves. The distribution of both values did not follow a normal distribution ($p<0.0001$, D'Agostino and Pearson omnibus normality test). We chose an arbitrary cut-off value of 10% which would select approximately 0.3% of all patients. Such a cut-off value could be conveniently incorporated into routine praxis in our laboratory.

Analysis of patients with more than 10% hyperchromic RBC

In the observation period hematograms from 32.226 individual patients were analyzed at the Clinic of Hematology of the University Hospital of Zurich. Out of these samples, 162 (0.50%) contained more than 10% hyperchromic RBC (Fig. 2). Review of patients' records revealed an acquired reason for this finding in 37 patients (22.8%): A cytotoxic treatment was the most frequent condition identified, explaining the elevated hyperchromic fraction of RBC in 27 patients; the 10 remaining patients summarized under the heading “other causes” included three with autoimmune hemolytic anemia (AIHA), three with severe plasma hypoosmolality, and one each with HELLP-syndrome (hemolysis, elevated liver enzymes, low platelets), an aortic valve defect, a massive myocardial infarction and recent surgery for aorto-coronary bypass. The group of patients with a “known congenital” explanation for the elevation of hyperchromic erythrocytes comprised 6 individuals (3.7%) including 5 patients with HS and one with hereditary cryohydrocytosis[12], based on current and previous hematologic records at our hospital. For 119 patients (73.5%), classified as "unknown", we found no explanation for the elevated hyperchromic fraction. In these cases we either did not have access to a medical history or did not find evidence for any of the known reasons for hyperchromasia. Interestingly, 29 of these 119 patients were found to have acute or chronic viral infection, including 23 patients infected with HIV. For 23 of the remaining patients the percentage of hyperchromic RBC had been documented normal in the past ("not persistent"); in these cases we therefore postulated

an acquired cause. Finally, for a group of 66 patients, the elevation of the hyperchromic fraction was unexplained and likely to be persistent; in all probability this group would include patients with undiagnosed hereditary RBC membranopathies ("possibly congenital"). Laboratory findings of the different groups are shown in Table 1.

Unexpectedly we found 8 patients suffering from epilepsy among the 119 patients with neither a known congenital nor an acquired membranopathy. 5 of these patients were classified as "not persistent", 2 as "possibly congenital" and one had a documented HIV-infection. Anticonvulsive treatment at the time of investigation could be confirmed in 6 of these patients by medical history including lamotrigine (2 patients), valproic acid (2 patients), carbamazepine (1 patient) and phenytoin (1 patient).

Patients of the "possibly congenital" group as well as several members of the other groups were contacted for further testing. Ektacytometry could be performed on 32 subjects; four of these were of the "known congenital" group (three HS and one cryohydrocytosis) and were aware of their condition. Their osmoscans are shown in Fig. 3B, illustrating a mild case of HS, two pronounced cases of HS and one case of genetically confirmed cryohydrocytosis[16]. Sixteen of the remaining 28 patients examined by ektacytometry showed osmoscans characteristic for HS with 14 displaying a mild and 2 a more pronounced form of HS (Fig. 3C). None of these patients had been diagnosed with HS before.

Next we compared osmoscans of these newly diagnosed patients with osmoscans of previously described HS patients[6] (Fig. 3D); the molecular defects in the latter individuals had been confirmed by SDS-PAGE demonstrating band 3 deficiencies (5 patients, black symbols) and spectrin/ankyrin deficiencies (10 patients, red symbols). Importantly, osmoscans of both groups showed virtually overlapping characteristics: i) a significantly decreased maximal deformability, ii) a lower surface to volume ratio, as indicated by a right shift of the left arm of the osmoscan and iii) an increase in MCHC (a loss of solutes) while reaching a non-deformable state at lower osmolality, apparent by a left shift of the right arm of the osmoscan. Therefore, it is not possible to differentiate HS resulting from different membrane defects by ektacytometry on whole blood. However, there is no doubt that the diagnosis for all patients whose osmoscans are shown in Fig. 3C and 3D is HS.

In addition to HS, our analysis also revealed other characteristic osmoscan patterns; for example a group of four patients with "possibly congenital" elevation of the hyperchromic RBC fraction showed osmoscans in which the left arm was virtually identical to controls, but the right arm was shifted to lower osmolalities (Figure 3E). This pattern is highly similar to the osmoscans of two patients with documented viral infection as shown in Fig. 3F. The underlying pathophysiological reasons underlying this osmoscan pattern are unclear; since such a pattern seems to accompany several viral diseases we refer to it as a "viral pattern".

Furthermore, the remaining four tested patients assigned to the "possibly congenital" group had unique osmoscans, one fitting the diagnosis of dehydrated stomatocytosis[17] (Fig. 3H, green line). The remaining three could not be assigned to a specific disorder (Fig 3H), pointing to as yet uncharacterized membranopathies or atypical presentations of known conditions. Interestingly, as shown in Fig. 3G, osmoscans of two patients with acquired reasons for hyperchromasia including one patient with AIHA and one after cytotoxic therapy also showed a characteristic pattern not shared by any other group. Importantly, none of the patients with >10% hyperchromic erythrocytes had a normal osmoscan pattern.

Discussion

The majority of patients with more than 10% hyperchromic erythrocytes feature an elevated MCHC, however this correlation is not strict: More than a third show an MCHC within the normal range. Vice versa, an elevation of the MCHC is not strictly associated with a marked elevation of hyperchromic erythrocytes, indicating a certain independency of these parameters. Our data argue for the clinical value of an elevated fraction of hyperchromic RBCs as a screening parameter. If the percentage of hyperchromic RBCs is elevated to more than 10% and an acquired condition can be excluded, a further follow up including tests for viral infections and hereditary membranopathies should be considered.

Among 32.226 patients undergoing a standard hematogram at the University Hospital of Zurich during a period of six months, we found 162 with more than 10% hyperchromic RBC. Patients were classified according to their medical history. In 23% of theses patients we found known acquired reasons and, unexpectedly, in 18% a viral infection. After excluding patients with only temporary elevations of the hyperchromic fraction, 66 patients (41%) of the original cohort remained as candidates for a congenital but so far undiagnosed RBC membrane disorder. In this study, 32 patients with an elevated fraction of hyperchromic erythrocytes were examined by ektacytometry and none of these patients revealed a normal pattern of RBC deformability. Among the patients with a possibly congenital condition we could investigate 24 by ektacytometry and found 16 osmoscans typical for HS (Fig. 3C). All of these patients presented without clinical symptoms of HS. Osmoscans allowed the classification into a mild form of HS (14 patients) and a pronounced form (2 patients). Comparisons of these osmoscans with those recorded from patients with known membrane protein deficiencies confirmed that all of these newly discovered patients have HS.

According to our data, the prevalence of HS might be higher than generally estimated. Assuming, that the patients tested by ektacytometry would be representative for the "possibly congenital" patient group, the number of HS positive patients in this group can be extrapolated to be 44 (67%). Adding the 5 patients with previously known HS, the estimated number of HS patients within the 162 patients with hyperchromasia greater than 10% would be 49 (30%). The prevalence of HS in our collective of 32'226 patients can be calculated to be at least 1:650. Since the sensitivity of our screening test "hyperchromasia >10%" is not known, an unknown number of HS patients might have been missed by our study and the prevalence of HS would be even higher. In the literature, the prevalence of HS is usually estimated to be around 1:2'000, »including the very mild or subclinical forms« [1]. Our data thus suggest an at least threefold higher prevalence, probably due to a large number of clinically silent cases of HS, which are never tested or missed by a more conventional analysis. However, it is not known, whether the

patient collective undergoing standard hematogram analysis at our institution is representative for the Swiss population.

Interestingly, our screening test using the percentage of hyperchromic RBC also frequently revealed patients with viral infections. In fact, of the 119 patients with neither a known congenital membranopathy nor a condition for an acquired hyperchromasia, 29 (24%) had a documented viral infection, typically hepatitis or HIV. This is a strong indication that these viral infections can cause hyperchromasia. To the best of our knowledge, this association has not been described previously; One explanation for our finding would be an inhibition of erythropoiesis by certain viral infections, resulting in an over aged RBC population. For older RBCs membrane flexibility and cell water content would be decreased[18,19], leading to an increase of the corpuscular hemoglobin concentration and of the fraction of hyperchromic cells. In agreement with this interpretation, the mean reticulocyte count was lowest in the group of patients with documented viral infections (Table 1), confirming the postulated a lower RBC turn over.

Our analysis suggests that a simple test for hyperchromasia can identify individuals with contraindications for blood transfusion, thus potentially improving the quality of blood donations. In our study congenital RBC membranopathies which are considered contraindications for blood donations by several institutions[2] were detected at a surprisingly high rate. Furthermore, some of the remaining patients suffered from viral infections including HIV and should also be excluded. However, any potential benefit of such an additional screening test can only be demonstrated by a prospective study. In our study, osmotic gradient ektacytometry has proven its reliability and sensitivity for the recognition and classification of RBC membrane disorders. The osmoscans of RBC from healthy donors only show minute variations (Fig. 3), whereas those of RBC from patients with an acquired or hereditary hyperchromasia show a broad spectrum of variance, depending on the cause of hyperchromasia. Importantly, some osmoscan patterns could not be attributed to any of the known RBC membranopathies, potentially pointing to as yet undiscovered red blood cell abnormalities or disorders. The considerably elevated spleen diameter (Table 2) found in patients of this group supports this hypothesis. A detailed follow up of these patients is in progress and might lead to new insights into RBC physiology. Taken together, our study suggests that a simple test for hyperchromasia can identify individuals with contraindications for blood transfusion. The use of this parameter could potentially improve the quality of blood donations.

Acknowledgments & Authorship Contributions

JWD and JSG performed experiments, analyzed results, made the figures and wrote the paper. JSG designed the research-plan. Hans Ulrich Lutz (Institute of Biochemistry, ETH Zurich) reviewed the data and the paper critically and provided the data of the SDS-PAGE proven patients with HS. Benjamin Misselwitz (Division of Gastroenterology, Triemli Hospital Zurich) reviewed the paper.

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Tables

Table 1. Basic characteristics of patient groups.

	units (Reference)	Acquired		Congenital	Unknown		
		Cytotoxic	Other		Not pers.	Poss. cong.	Viral
No of patients		27	10	6	23	66	29
Sex	M:F	15:12	5:5	4:2	20:3	48:19	27:2
Age	years	54 (18-90)	54 (20-89)	36 (20-44)	60 (25-104)	41 (21-84)	46 (26-73)
Hemoglobin	g/dl (11.7-17.0)	11.5 (7.3-15.5)	11.7 (6.5-15.2)	14.8 (13.0-17.2)	15.3 (13.0-17.2)	14.3 (8.1-17.6)	14.4 (4.8-17.6)
Reticulocytes	‰ (4-25)	33.5 (0.0-104.2)	47.8 (10.7-151.6)	23.4 (15.0-39.4)	24.3 (5.7-56.4)	29.4 (9.4-123.0)	18.7 (4.8-44.3)
MCHC	g/dl (31-36)	36.4 (27.5-38.3)	37.0 (34.8-39.1)	36.4 (28.3-39.4)	37.1 (36.0-38.3)	37.0 (26.7-39.8)	37.2 (35.6-38.4)
MCV	fl (80-100)	91.0 (82.6-101.0)	85.7 (80.6-92.8)	82.8 (76.7-85.9)	86.2 (79.0-98.4)	85.2 (25.3-98.4)	90.5 (39.6-101.1)
MCH	pg (26-34)	33.4 (29.9-38.4)	31.7 (29.6-34.9)	31.6 (28.7-33.1)	31.9 (29.5-36.1)	32.5 (27.5-68.7)	34.4 (29.3-43.8)
LDH	U/l (240-420)	477 (224-791)	1120 (400-4656)	449 (357-546)	531 (240-1980)	427 (236-791)	486 (66-790)
Bilirubine	µMol/l (<21)	93 (7-819)	39 (6-109)	33 (27-39)	15 (4-31)	15 (5-36)	33 (9-80)
Neutrophils	10^9/l (1.4-8.0)	5.90 (0.90-20.30)	12.65 (2.10-47.80)	5.57 (3.00-8.80)	5.07 (1.20-19.10)	5.58 (0.50-46.30)	3.25 (1.20-6.70)
Lymphocytes	10^9/l (1.5-4.0)	1.02 (0.09-2.80)	6.70 (0.40-54.70)	2.36 (1.19-5.24)	1.37 (0.34-4.16)	2.53 (0.54-43.10)	1.82 (0.28-3.44)
Thrombocytes	10^9/l (143-400)	216 (35-471)	173 (34-310)	386 (196-851)	207 (71-366)	243 (32-547)	186 (28-366)

The mean and the range of the observed values are indicated.

Table 2. Characteristics of patients studied by ektacytometry.

	(Reference)	Viral	Mild HS	Pronounced HS	Unknown
No of patients		4	14	2	4
Spleen diameter	mm (<120)	130 (122-137)	140 (130-155)	136 (101-170)	208 (185-230)
Reticulocytes	‰ (4-25)	20 (12-25)	25 (18-44)	64 (38-90)	61 (26-123)
Hemoglobin	g/dl (11.7-17.0)	15.4 (13.5-16.2)	15.2 (13.9-17.6)	10.8 (8.7-12.8)	13.6 (8.1-16.5)
LDH	U/l (240-420)	382 (314-449)	373 (343-398)	531 (492-570)	543 (308-778)
Bilirubine	µmol/l (<21)	22 (10-34)	14 (5-36)	30 (28-31)	14 (7-21)

The mean and the range of the observed values are indicated

Figures

Figure 1: Plot of MCHC as a function of the fraction of hyperchromic RBC.

All patients undergoing standard hematogram analysis during a period of 2 months at our institution were included. The vertical lines indicate the upper and lower limits of normal (>36g/dl and <31g/dl, respectively). Similarly, the lower and upper reference values for the fraction of hyperchromic RBC (2% and 10%, respectively) are indicated as horizontal lines. The number of patients in each section of the plot is given.

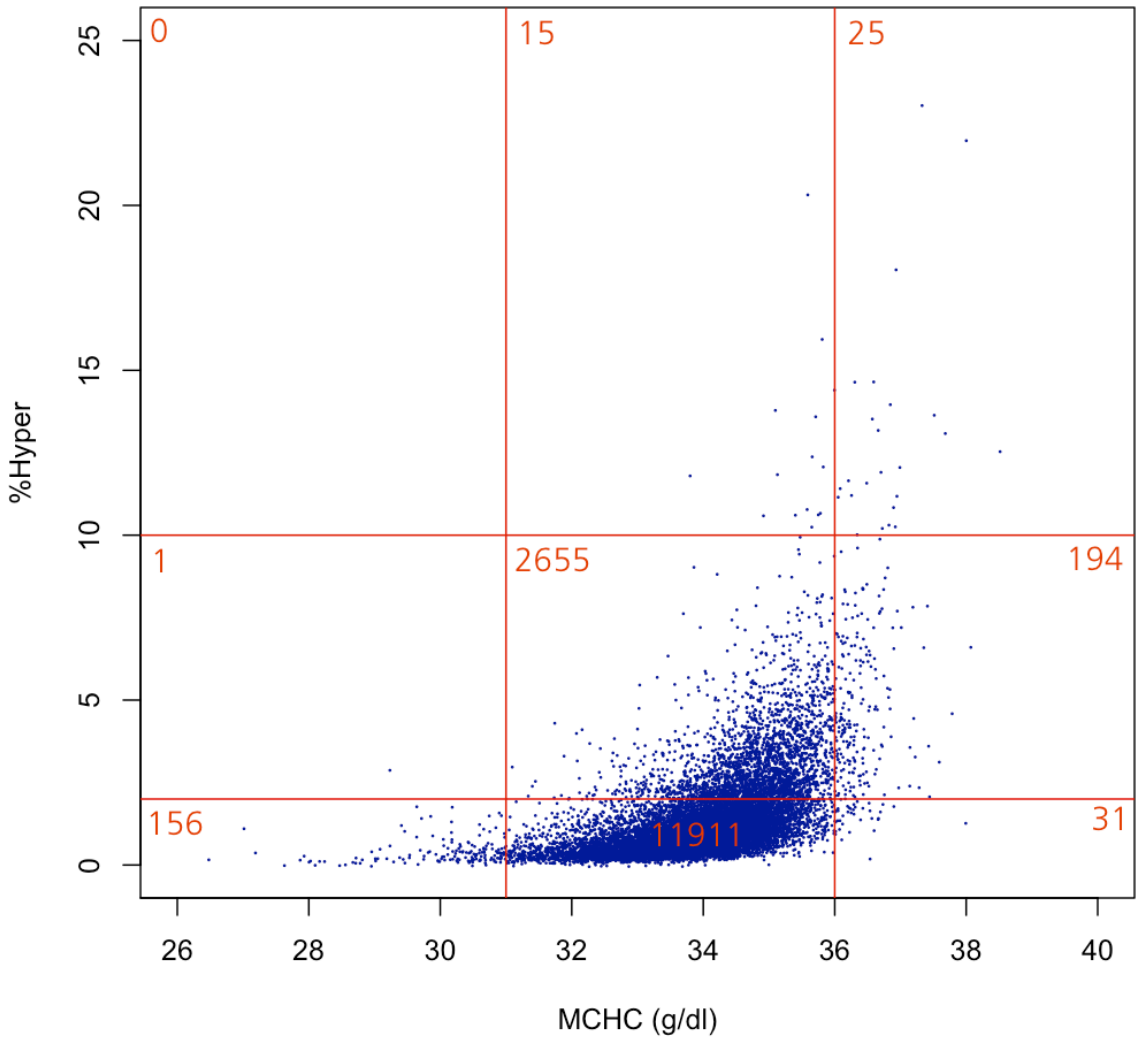


Figure 2: Study design.

During a period of six months all hematograms acquired were reviewed. For samples with more than 10% hyperchromic RBC patient records were analysed retrospectively. Patients with a known RBC disorder or a known condition leading to an elevation of hyperchromic RBCs were thus classified as having a "known congenital" or an "acquired" hyperchromasia. All other patients ("unknown") were separated into patients "with documented viral infection" and without. Patients with a transient elevation of hyperchromic RBCs were excluded from follow up "not persistent", the condition of the remaining patients was regarded as "possibly congenital". Of these, 24 could be further examined by osmotic gradient ektacytometry. The resulting osmoscans followed four distinct patterns: (mild HS (14), pronounced HS (2), viral pattern (4), unknown patterns (4)).

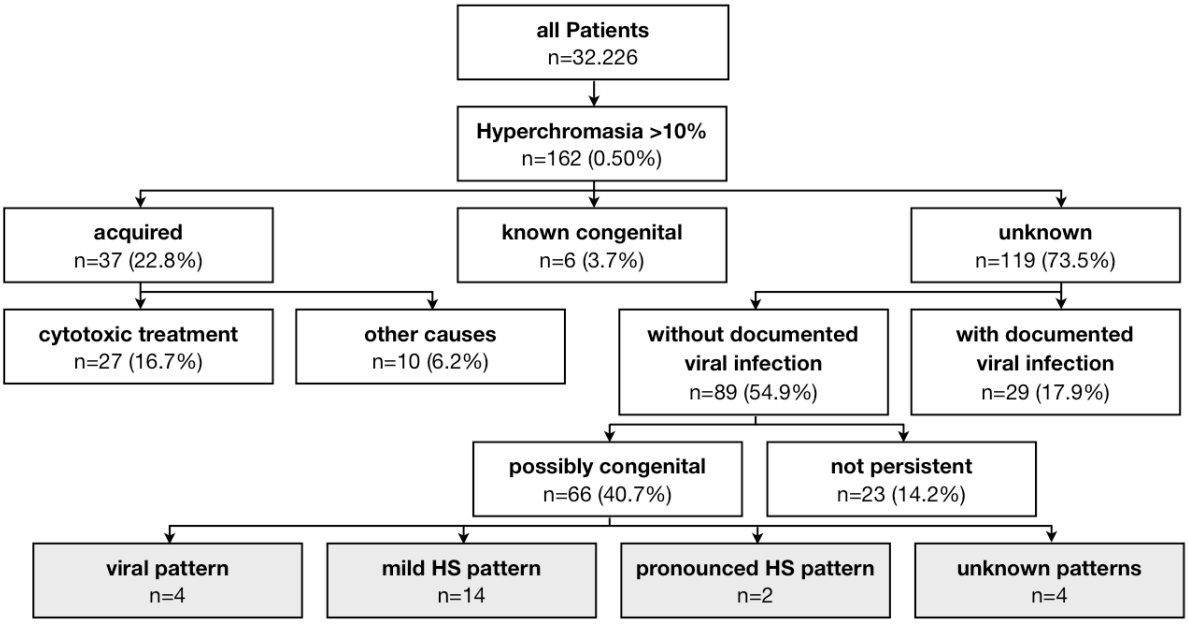
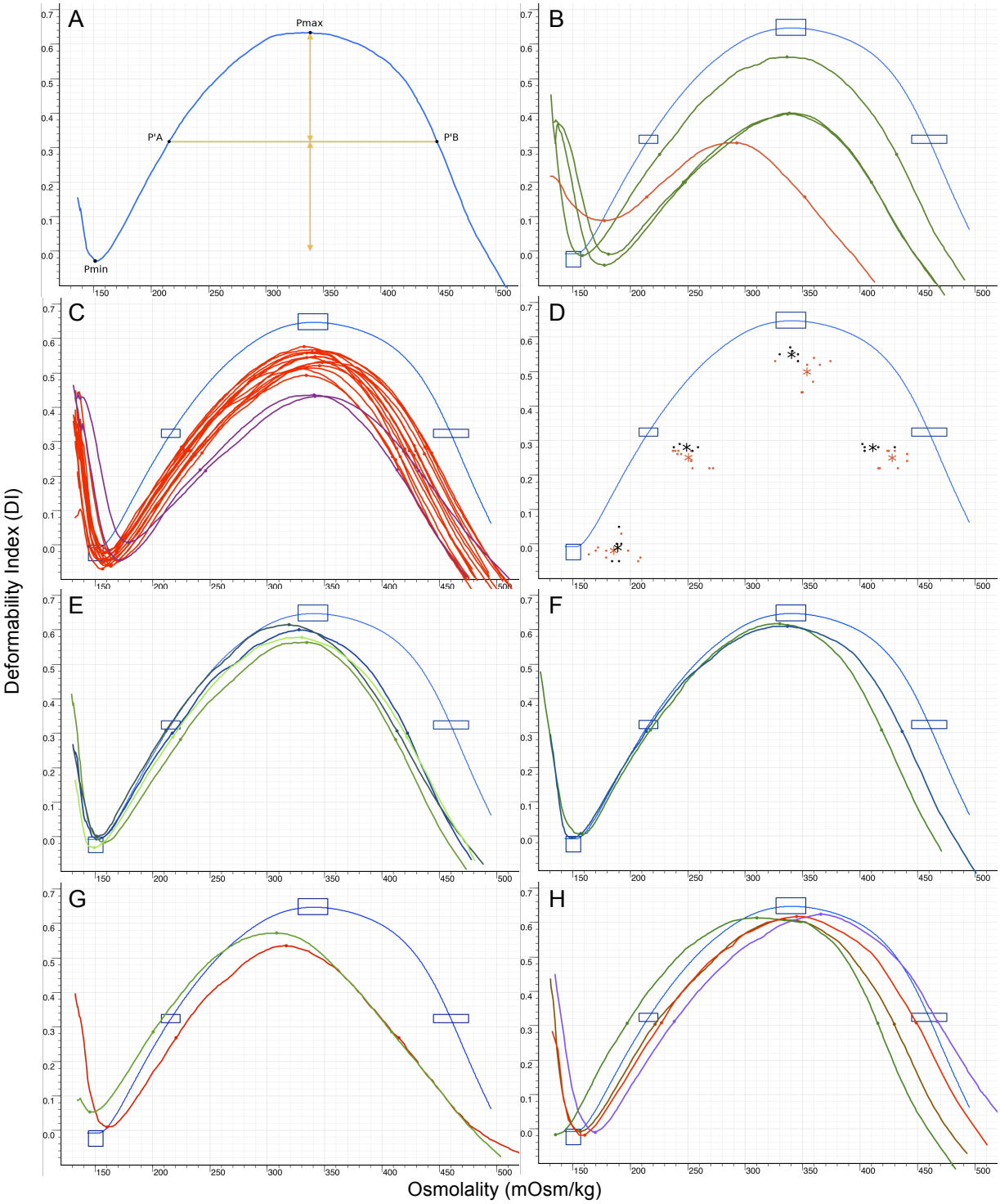


Figure 3: Osmoscans.

(A) Characteristic points of osmoscans. Pmin is the point of 50% hemolysis. Pmax is the point of maximal deformability. On the hyper- and hypoosmolal branches of the osmoscan, the osmolality at which half the maximal deformability (1/2 DI of Pmax) is reached are defined as P'A and correspondingly as P'B. **(B) Patients with a known hereditary membranopathy of the RBC.** Patients with known membranopathies were studied by ektacytometry. Three patients show different forms of HS (green), one patient has hereditary cryohydrocytosis (red). The ektacytometric features of HS consist of Pmax with deformability well below the normal range though at a normal osmolality, and often an increased osmolality of Pmin, indicating a diminished osmotic resistance. The osmolality of P'A is increased, whereas that of P'B is shifted to hypoosmolal conditions. Cryohydrocytosis has a different etiology, osmoscans show a reduced maximal deformability, a dramatically limited flexibility at high osmolality such that all characteristic points are shifted to hypoosmolality except for Pmin, which is shifted to hyperosmolality, again indicating a decreased osmotic resistance. For comparison, we added the mean curve of 30 healthy donors (blue) into all the following osmoscans. The blue boxes indicate the normal range of the four characteristic points Pmax, Pmin, P'A and P'B (double standard deviations). **(C) Osmoscans of patients with "possibly congenital" elevation of the hyperchromic RBC-fraction revealing a pattern of HS.** Fourteen patients' osmoscans (red) reveal a mild form of HS, whereas 2 (purple) impress as more pronounced forms of HS. All osmoscans show the typical features of HS with a decreased maximal deformability and a loss of cellular solutes. The osmolality at which Pmin is reached is for the majority of cases only slightly higher than that of controls. This implies a minute reduction of the osmotic resistance and probably explains why all of these patients were asymptomatic. **(D) Osmoscans of HS patients with SDS-PAGE confirmed membrane protein deficiencies.** The black points originate from 5 patients with band 3 deficiency, the red points from 10 patients with spectrin/ankyrin deficiency. The averages of the characteristic points for the two types of disorders are marked by asterixes in the corresponding colors. **(E) Osmoscans from patients with "possibly congenital" elevation of the hyperchromic RBC-fraction revealing a viral pattern.** Pmin and P'A are in the normal range, Pmax is slightly diminished and shifted to hypoosmolality, whereas P'B shows a clear shift to hypoosmolality. This solitary shift is characteristic for aged RBC that have lost solutes prematurely. The normal osmolality of Pmin indicates a normal osmotic resistance of the RBC from these patients. **(F) Osmoscans from two patients with documented viral infection.** These osmoscans are very similar to those shown in Fig. 2E. **(G) Osmoscans from two patients with acquired membranopathies.** One patient was undergoing cytotoxic treatment (green curve) and one patient had autoimmune hemolytic anemia (AIHA), shown in red. **(H) Osmoscans from four patients with "possibly congenital" elevation of the hyperchromic RBC-fraction revealing unknown patterns.**

We could not assign these osmoscans to a specific RBC membranopathy, despite all curves suggest some sort of membrane disorder. The green curve may fit to the pattern of a mild form of dehydrated stomatocytosis, with all characteristic points being shifted to hypoosmolality and the maximal deformability being only slightly diminished. The blue curve is the only curve of all measured samples that shows P'B at a higher osmolality as the mean of normal controls. The red curve possibly shows a borderline type of HS, with a slightly diminished osmotic fragility (Pmin) and a slightly diminished maximal deformability (Pmax). The brown curve shows a pattern reminiscent of a viral infection, except for the right shift of the left arm of the osmoscan.


A
Pmax
P'A
P'B
Pmin
B
C
D
E
F
G
H
Deformability Index (DI)
Osmolality (mOsm/kg)

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